Development of Co-cultured Microfluidic Platform for Mimicking the Blood-Brain Barrier

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Jiwon Yom

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Development of Co-cultured Microfluidic Platform for Mimicking the Blood-Brain Barrier

Approved by:

Mentor
YongTae Kim, PhD.
Assistant Professor
The George W. Woodruff School of Mechanical Engineering

Date: 4/25/17

Young Jang, PhD.
Assistant Professor
School of Biological Sciences

4/28/17
Date:

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Abstract

A blood-brain barrier (BBB) is a biochemical, physical barrier found in the brain vasculature that only allows selective transport of molecules in order to protect the brain from potential damages. Due to its complex structure of cellular arrangement, there is a lack of a physiologically relevant BBB model that could be used to test the efficacy of drugs that treat brain cancers, such as glioblastoma multiforme. Therefore, this study aims to introduce an *in vitro* BBB model on a microfluidic platform that captures the dynamic nature of BBB, mainly permeability of the BBB. The proposed BBB model, or BBB on a chip (BBBoC), incorporates human primary cells that comprise a human BBB, including human brain microvascular endothelial cells (HBMECs), human brain vascular pericytes (HBVPs), and normal human astrocytes (NHAs). To validate the proposed BBB model, this study tests for permeability of different combinations of the primary brain cells to observe the effect of cellular composition on permeability. Finally, an effective in vitro BBB model is introduced.

Background

Glioblastoma multiforme (GBM) is one of the most frequent, fatal forms of primary brain tumor.[1,10] GBM is characterized and induced by overexpression, activation, and dysregulation of various membrane receptors, signaling pathways, and other factors.[2] At the present time, the two-year survival of the patients diagnosed with GBM is approximately 28% with median survival of 16.5 months.[9] The current standard procedure for treating GBM is invasive and includes resection followed by chemotherapy, which aims to eliminate the tumor.[2] However, due to the infiltrative nature of GBM, a gross-total resection (GTR) often does not have a significant influence on a microscopic level.[11] It is also important to note that GTR will not always be a primary option because GTR is often limited in size as brain is a vital organ. As a result, chemotherapy is used as a follow-up or an alternative procedure. Although many combinations of drugs have been introduced, there is a lack of 'personalized' therapy, and the level of cytotoxicity of the new strategies has yet to be determined.[2] The drug screening for GBM is often difficult to evaluate due to the presence of blood-brain barrier (BBB). BBB is a complex structure in the brain that consists of endothelial cells, pericytes, astrocytes, neurons and microglia, which comprise the neurovascular unit (NVU).[3] The interactions between the different cell types contribute to the specialization of the endothelial cells that regulate passage of molecules in and out of the brain.[4] Thus, investigating the transport pathway through the BBB under GBM condition is crucial in order to not only enhance the efficacy of the drugs but also potentially find better candidates to treat GBM.

In pursuit of gaining insight into the complexity that surrounds the BBB, there have been various efforts to model BBB *in vitro*. At an earlier stage, the role of endothelial cells was viewed more significant to other types of cell that constitutes the BBB. Monoculture of brain endothelial cells was viewed as a sufficient model to study the transport phenomenon through the BBB.[4] The endothelial monolayer was cultured on top of a semi-porous membrane and under static condition.[4] Although these models may enlighten us on the response of isolated endothelial cells to a particular drug, the results are questionable due to the lack of physiological relevance in their design, such absence of dynamic flow and NVU.

In the earlier example, the model does not incorporate fluid flow. Wolff et al. and many authors argue that this creates significant problem as the endothelial cells are cultured under less

favorable environment, as shown by lower transendothelial electrical resistance (TEER) values compared to those of the cells cultured under shear stress.[4] Santaguida et al. even go further and claim that the static model is flawed as the absence of shear stress reduces the tightness, shown by TEER values, and the permeability to polar molecules through the monolayer compared to the values *in vivo*.[6] Although agreeing on these drawbacks in using static model, Wilhelm et al. raises another view on the static model by pointing out that the static model is inexpensive and is easy to handle, and therefore, for the sake of high-throughput screening, the simple endothelial monolayer BBB model is quite adequate.[5] It seems agreeable that the simple model would drastically remove grossly inadequate candidates and thus save time and money. However, because the integrity of the monolayer is suboptimal in the absence of shear stress, the simple model may further complicate, rather than simplifying, the candidate selection. In addition, for the sake of researching the BBB in the context of GBM, the monolayer model would not be suitable as the cancer would further induce leaky vasculature and disrupt the integrity of the monolayer, and therefore the model would not produce any meaningful results.

In an effort to increase physiological relevance, many researchers adopted microfluidic 'organ-on-a-chip' approach to introduce fluid flow. Santaguida et al. describes one microfluidic model produced by Prabhakarpandian et al., which has two compartments segregated by a porous membrane and is able to induce microcirculation within the compartments.[6] Although praising the design incorporates shear stress through fluid flow, Santaguida et al. also points out that the immediate effectiveness of microfluidic model is hard to be determined as measurements of TEER or other markers are challenging on microfluidic platform.[6] In the same regards, Wilhelm et al. expresses that the microfluidic model is not well established.[5]

The other pressing issue about the simple monolayer is the fact that it is lacking physiological relevance to the human BBB. The BBB is comprised of many cell types, such as astrocytes and pericytes.[3] The endothelial monolayer itself may exhibit the properties of the BBB due to tight junctions; but, in the case like GBM, a deeper insight into the interactions inside the NVU is critical. While some tri-culture models have been proposed, most of the cases of tri-culture are done in a static environment. There are some cases where co-culture was done on a dynamic environment, such as microfluidic platform, but is typically limited to endothelial cells and astrocytes.[7,8]

Besides the previous efforts to produce BBB models, there have been efforts to produce GBM models. For example, Fan et al. utilized hydrogel-based microwells where GBM cancer cells were seeded to produce GBM spheroids.[12] Although this model allowed three-dimensional culture of GBM cancer cells, this model also exhibits the same issues of static environment and lack of physiological relevance. Other studies include *in vivo* models, such as murine GBM model. Katz et al. injected RCAS-PDGF avian retrovirus into healthy mice subjects, thereby performing a somatic gene transfer of an oncogene PDGF to cause genetic aberrations, such as loss of tumor suppressor on glioma formation, and consequently inducing GBM.[22,25] Despite the fact that both authors directly used cancer cells, their relevance to the human GBM physiology is questionable.

Introduction

Glioblastoma multiforme (GBM) is the most malignant astrocytic tumor [1,11] and approximately 10,000 new cases of GBM are diagnosed per year.[10] Currently, the standard procedure for GBM includes resection followed by chemotherapy and chemoradiotherapy, which aims to disrupt receptor-ligand interactions or signaling pathways in the cancer development.[2,10] Yet, the presence of blood-brain barrier (BBB) poses a challenge to drug delivery in chemotherapy due to the complex structure and the high selectivity.[3,5] Various *in vitro* studies have focused on drug delivery through the BBB in order to increase the efficacy of systemically administered drugs. However, the proposed BBB models in the previous studies are lacking in physiological relevance due to their static nature[5,14] and the absence of co-culture of the various human brain cells that compose the BBB.[7,8] There is no adequate BBB model that is suitable to evaluate the drugs used to treat GBM despite the increasing demand for chemotherapy. Therefore, the goal of this study is to develop a physiologically relevant BBB model.

In order to address the issues of co-culture and static environment, this study aims to utilize microfluidic technology to reproduce the *in vivo* conditions of the human BBB.[22] The BBB model proposed in this study is a double-layer microfluidic device that is composed of two different compartments segregated by a semi-permeable membrane. In this model, the top compartment was seeded with human brain microvascular endothelial cells (HBMECs) to mimic human brain capillaries.[15,16,17] In addition, to construct the *in vivo* structure of the BBB, human brain vascular pericytes (HBVPs) will occupy the bottom side of the membrane and the bottom compartment will be filled with normal human astrocytes (NHAs) in a hydrogel.[16,17,18,19] Because the membrane is separating the two compartments, the HBVPs seeded on the bottom side of the membrane are in contact with the NHAs seeded in the bottom compartment. In this setup, HBMECs are also able to interact with both HBVPs and NHAs. Such proximity would allow the interactions between each cell type observed *in vivo*.[17,19,20,21] Moreover, the double-layer device has microfluidic channels which allows to model the physiological dynamic conditions *in vivo*.

Then, the proposed BBB model was tested for validation. To validate the model, permeability test was conducted with FITC-Dextran. It was reported that tri-culture of porcine primary pericytes, astrocytes and endothelial cells increases the integrity of endothelial

monolayer compared to the monolayer under monoculture.[28] As BBB is a conserved physiology between pigs and human, tri-culture of HBMECs, HBVPs and NHAs should also demonstrate much lower permeability compared to that from HBMEC monoculture. In addition, immunostaining and confocal imaging was performed to visualize the cells, which visibly confirmed that the changes in permeability resulted from the difference in the cellular components. With these results, the BBBoC model was validated.

There is a clear lack of physiologically relevant model that is suitable for GBM drug research. In order to address these issues, this project focuses on producing an *in vitro* BBB model to mimic the *in vivo* conditions of BBB on a microfluidic platform. Hence, a novel BBB-on-a-Chip (BBBoC) is introduced. By incorporating human primary microvascular cells in the model, BBBoC addresses lack of a physiologically relevant model needed for researching and developing drugs. The tri-culture will be conducted in a microfluidic device under dynamic conditions to induce shear stress and to illustrate fluid flow in the BBB. As a result, this project presents an *in vitro* human BBB model that can effectively capture the *in vivo* physiology of BBB and thus provide an accurate platform for drug screening.

Material and Methods

Microfluidic Device Fabrication

A photoresist was designed with SolidWorks and used in the process of producing SU-8 wafers for upper and lower channels. PDMS was (SLYGARD 184 Silicone Elastomer Kit) used to perform PDMS soft-lithography, thereby producing upper and lower channel PDMS layers. The lower channel PDMS layer was bonded to a 1mm glass slide through plasma bonding. With the same bonding procedure, membrane (SterliTech 8.0 micron, 25mm PCTE membrane filters) coated with 5% APTES was placed on top of the lower channel PDMS layer, and the upper channel was placed on top of the membrane. The completed microfluidic devices were placed in the oven at 80℃ for 2 days to induce hydrophobicity inside the microfluidic channels.

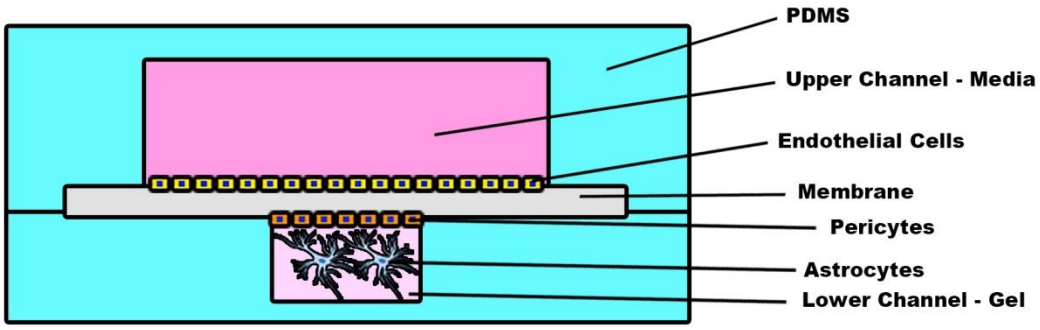

Figure 1. Schematic view of BBBoC. Cross-sectional view of the device is shown in order to visualize the composition of BBBoC. As it can be seen in the figure, the endothelial monolayer is formed in the upper channel to represent luminal side of BBB, while pericytes and astrocytes are surrounding the endothelial monolayer with membrane as the structural support.

Cell Culture

Human Brain Microvascular Endothelial Cells (HBMECs; ScienCell) were cultured with Endothelial Cell Medium (ECM; ScienCell) with 5% FBS, 1% endothelial cell growth supplement and 1% penicillin/streptomycin solution. Human Brain Vascular Pericytes (HBVPs; ScienCell) were cultured with Pericyte Medium (PM; ScienCell) with 2% FBS, 1% pericyte growth supplement and 1% penicillin/streptomycin solution. Normal Human Astrocytes (NHAs; ScienCell) were cultured with Astrocyte Medium (AM; ScienCell) with 2% FBS, 1% astrocyte growth supplement and 1% penicillin/streptomycin solution. The cells were cultured at 37 ℃ supplied with 5% CO_2. Due to concerns for differentiation, these primary cells were not allowed to exceed passage 6.

Seeding Procedure

For sterilization, the channels of the microfluidic devices were flushed with 80% ethanol at 50μL/min and exposed to UV light for an hour. The sterilization was followed up by washing process, in which PBS was sequentially used to flush the microfluidic channels. Both sides of the membrane were coated with 5mg/mL fibronectin for 1 hour to facilitate seeding process. Mixed media (ECM:AM=1:1) was used to flush out the coating solution and fill up the channels. When the cell population is 80% confluent, the cells were trypsinized and centrifuged at 160*g for seeding. Each cell type was prepared at $1x10^6$ cells/mL with respective media, with the exception of NHA which was prepared in Matrigel (Corning).

Total of 4 treatment groups are tested in this study, which includes a control (gel only), a monoculture (HBMEC) model, a coculture (HBMEC+HBVP) model, and a triculture (HBMEC+HBVP+NHA; BBB) model. For control, only the lower channel of the microfluidic device is filled with gel. For monoculture model, only HBMECs are seeded on the upper channel. For coculture model, HBVPs are seeded on the bottom of the membrane first followed by HBMEC seeding in the upper channel. For triculture model, HBVPs were seeded first on the bottom of the membrane, followed by NHA in the bottom channel and then HBMEC in the upper channel. In this paper, triculture refers to the BBBoC. After seeding, the microfluidic devices were incubated at 37 ℃ supplied with 5% CO_2 for 2 days before testing permeability.

Permeability

EVOS microscope (Life Technologies) was used to take time-lapse images for 10 minutes. A region of interest (ROI) was defined on the lower channel focal plane. FITC-dextran (4k; Sigma) was prepared at 10mg/mL in DMEM and was introduced to the upper channel or the lumen side of the BBBoC. Before administering FITC-dextran solution, standard curve was produced with serial dilutions. Once FITC-dextran solution was introduced, the time-lapse initiated. The images were analyzed through ImageJ to determine the change in intensity of fluorescence to calculate permeability with the following equation[8]:

$$\mathrm{P} = \frac{J_s}{AC_L}$$

where J_s is the solute flux calculated from the change in fluorescence intensity with the standard

curve, A is the effective surface area for solute transport, and C_L is the concentration of FITC-Dextran at the luminal side (top compartment).

Immunostaining

Immunostaining was performed in order to visualize each cell type. The samples were fixed with 7% paraformaldehyde, permeablized by 1% Triton-X, and blocked by 2% BSA. For HBMEC, VE-cadherin was stained, which was tagged with Alexa 647 secondary antibody. HBVP was stained with FITC-tagged αSMA antibody. GFAP was targeted on NHA and was stained with Alexa 598. In addition, ZO-1 in HBMEC was stained with FITC-tagged ZO-1 antibody.

Confocal microscopy (Zeiss) was performed in order to visualize the changes in the gap junction expression in each model. As the permeability of the model is determined by gap junctions expressed in HBMEC monolayer, ZO-1is stained for all of models to observe for different levels of gap junction expressions in HBMEC monolayer across the models.

Statistical Analysis

Student two-tailed t-test is performed at $\alpha=0.05$ to determine the statistical significance of the difference in permeability between the test groups. In addition, one-way ANOVA at $\alpha=0.05$ will be performed in order to validate the significance in the difference of permeability values among all models. Based on the p-values obtained from the tests, each comparison between two of the test groups will be categorized in to the following groups: *($p<0.05$), **($p<0.005$), ***($p<0.0005$), and ****($p<0.00005$).

Results and Discussion

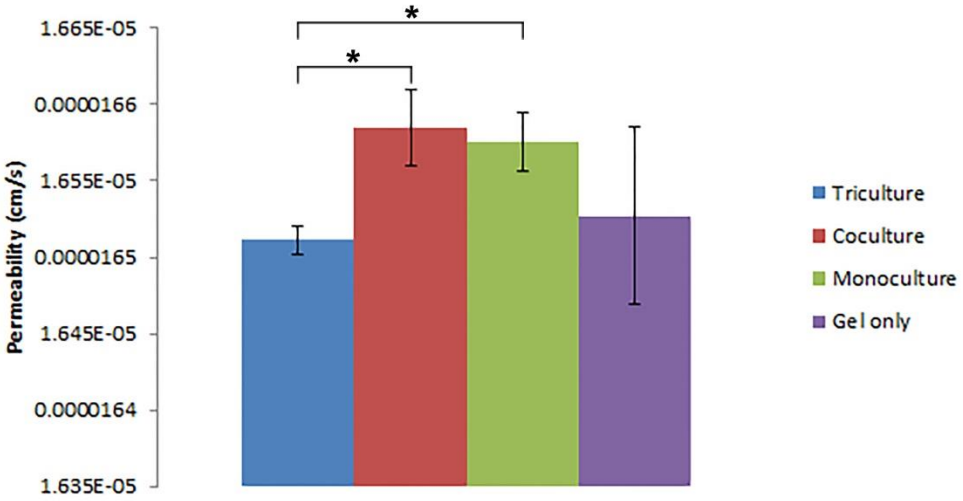

Figure 2. The Effect of Culture Condition on the Permeability of Respective Models. FITC-Dextran (4k) was applied to all models. The permeability value for triculture was 1.6511×10^{-5} cm/s, while coculture and monoculture model showed permeability coefficient of 1.6585×10^{-5} cm/s and 1.6576×10^{-5} cm/s, respectively. Student's unpaired, two-tailed t-tests showed that the permeability of triculture was significantly lower than those of coculture and monoculture models. ($p=0.0167$ for coculture; $p=0.0127$ for monoculture) However, there was no significant difference between the permeability values of triculture and blank models. ($p=0.724$) One-way ANOVA performed for all models produced no significant result. ($p=0.149$)

As shown in Figure 2, the triculture model exhibited the lowest permeability value among other models. Statistical analysis validated the significance in the lower value of permeability under triculture condition compared to those under coculture and monoculture conditions. As BBB is characterized by a special barrier structure that is comprised of various microvascular cell types that leads to significantly low permeability, the fact that the triculture model demonstrated the lowest permeability shows the model's physiological relevance to *in vivo* conditions. Thus, it can be concluded that BBBoC is an effective *in vitro* model for human blood-brain barrier.

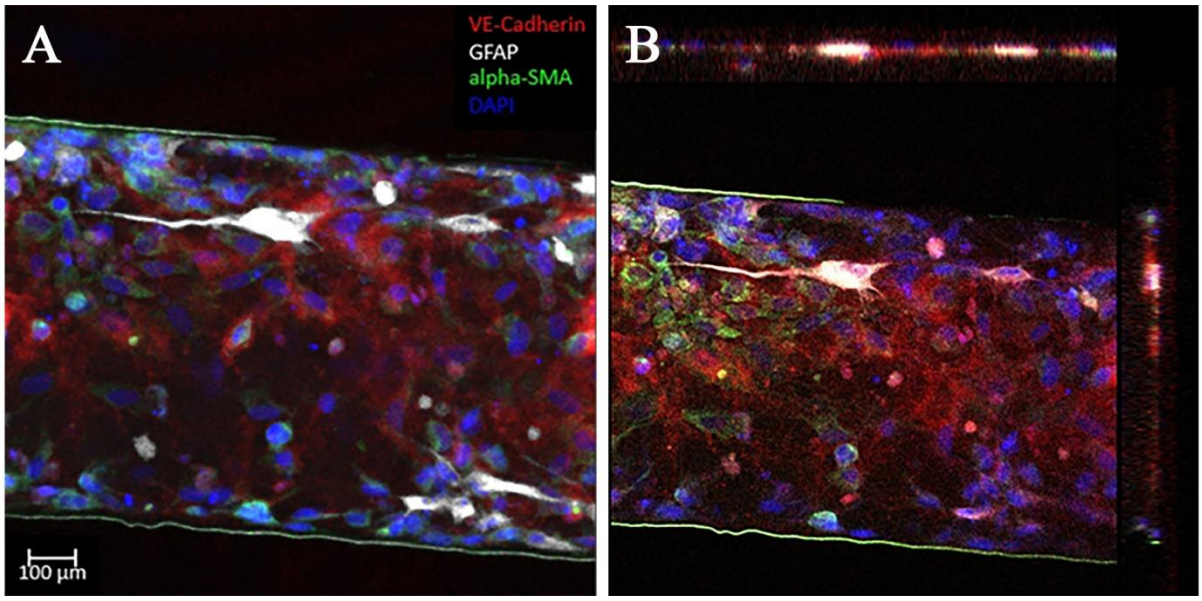

Figure 3. Immunostaining of BBBoC. The triculture was maintained for 3 days in order to allow endothelial cells to express adhesion and tight-junction proteins. Standard immunostaining procedures were performed, where VE-cadherin was stained in HBMEC, αSMA in HBVP, and GFAP in NHA. DAPI was used to stain the nucleus in all cell types. (A) Enhanced image of BBBoC. (B) Orthogonal views of BBBoC. As the orthogonal views show that cells fluoresce on a single plane, it is evident that all cell types are in contact, forming a blood-brain barrier.

However, as there was no significant difference between the blank and the triculture models, it is difficult to conclude that the triculture condition lowers the permeability. One of the reasons why the permeability change is not significant may arise from the fact that the astrocytes secrete matrix metalloproteases (MMPs) that delaminate the gel used to fill the lower channel.[31] In other words, it is possible that the resistance to diffusion for FITC-Dextran molecules is significantly reduced in the triculture condition as the MMPs had decomposed the gel in the lower channel. Thus, the permeability of FITC-Dextran was significantly enhanced in BBBoC triculture model. As a result, the difference between the permeability values of the triculture and the blank models became insignificant.

As it had been reported in previous studies, the level of tight-junction protein expressed in endothelial cells determines the degree of permeability of the model. To compare the level of tight-junction protein expressed, ZO-1 in HBMECs were stained with FITC-tagged ZO-1 antibody. The results are illustrated in Figure 4, which clearly shows that there is an increased

level of ZO-1 expression in the triculture model. This result corresponds to the results illustrated in Figure 2, where the permeability of the triculture model was the lowest.

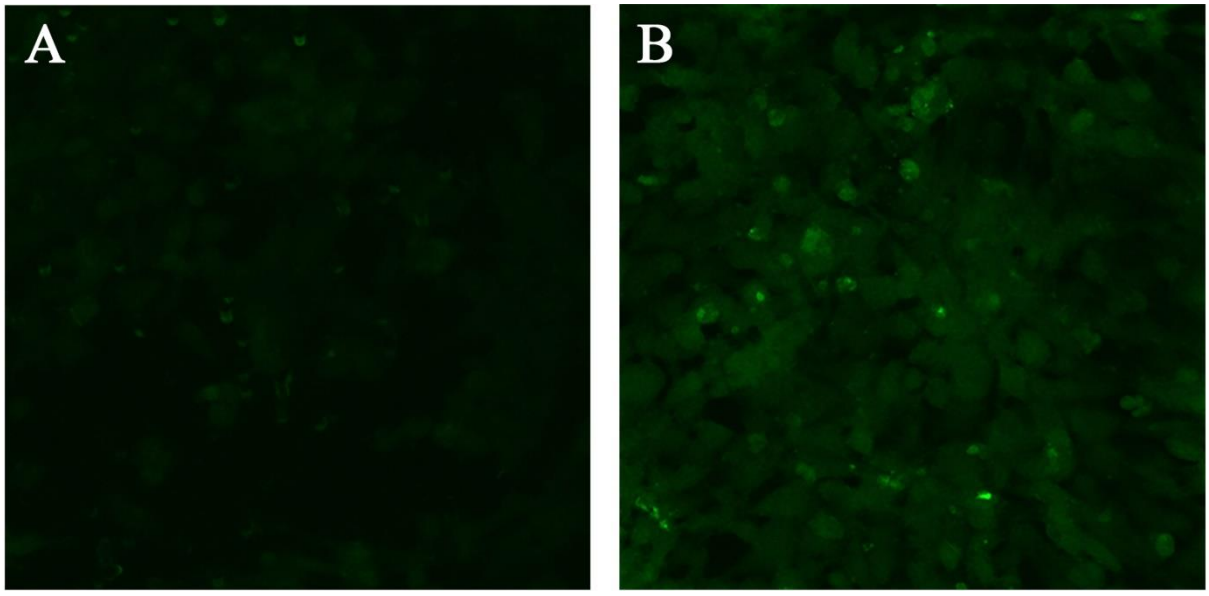

Figure 4. Immunostaining for ZO-1 tight-junction protein. (A) ZO-1 staining for coculture condition. (B) ZO-1 staining for triculture condition.

In addition, one-way ANOVA showed no significance in the permeability values of all models, meaning that there was no clear distinguishable effect of cell cultures, especially endothelial cells, on the permeability of the models. Thus, the results from ANOVA disagree with the results illustrated in Figure 2 and 4. However, because the level of ZO-1 expression was much more upregulated in the triculture compared to coculture, it is believed that the model was not cultured for long enough period of time for endothelial cells to form tight-junctions completely, which resulted in statistically insignificant difference among the permeability values for all models. In other words, there is a possibility of partial monolayer formation that must be evaluated in order to confirm that no area was significantly permeable to FITC-Dextran molecules, causing the permeability to incline.

Conclusion and Future Works

Based on the above stated results, the introduced BBBoC can be viewed as a physiologically relevant *in vitro* BBB model that mimics the human BBB *in vivo*. However, there are potential improvements that could be implemented. For instance, advanced techniques, such as qPCR or western blot, could have been used to yield more quantitative results that quantify the level of tight-junction protein expression so that the statistical significance level could be determined. In addition, as the purpose of the proposed BBB model lies in drug screening for GBM, further modification will be made to the BBB model to illustrate GBM conditions. The BBB model will be cultured with GBM-conditioned media in order to induce GBM conditions.[24] The BBB model under GBM condition can be validated with permeability assay. The GBM-conditioned BBB model should report significantly higher permeability compared to that of the BBBoC because the GBM condition perturbs the tight junction in the endothelial monolayer. Such will be visually confirmed with immunostaining Claudin-1, Claudin-5 and Occludin on normal and GBM-conditioned BBB models will be conducted. As it is reported that Claudin-1, Claudin-5 and Occludin, proteins present in tight junction, are significantly reduced in GBM, the results from immunostaining should positively correlate with permeability test results.[27] Consequently, an effective GBM-conditioned BBB model will be achieved. As a result, true *in vitro* model for developing GBM drugs will be developed.

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